MECHANISTIC EVALUATION OF RED ALGAL EXTRACTS THAT SLOW AGING

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MECHANISTIC EVALUATION OF RED ALGAL EXTRACTS THAT SLOW AGING

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LIST OF SYMBOLS AND ABBREVIATIONS

ABTS	2,2’-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
AO	Antioxidant
$CDCl_3$	Deuterated Chloroform
DMSO	Dimethyl Sulfoxide
DMSO-d_6	Deuterated Dimethyl Sulfoxide
ELSD	Evaporative Light Scattering Detector
FAME	Fatty Acid Methyl Ester
FUDR	5-Fluoro-2’-deoxyuridine
GC-MS	Gas Chromatography-Mass Spectrometry
HCl	Hydrochloric Acid
HPLC	High Performance Liquid Chromatography
LC-MS	Liquid Chromatography-Mass Spectrometry
mTOR1	molecular Target of Rapamycin Complex 1
NMR	Nuclear Magnetic Resonance
ROS	Reactive Oxygen Species
TLC	Thin Layer Chromatography
Trolox	6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid

SUMMARY

Aging results from an accumulation of damage to macromolecules inhibiting cellular replication, repair, and other necessary functions. Damage may be due to environmental stressors such as metal toxicity, oxidative stress caused by imperfections in electron transfer reactions, or other metabolic processes. In an effort to discover medical treatments that counteract this damage, we have initiated a program to search for small molecule drugs from natural sources. We have identified marine red algae as a source of natural products that slow aging of the invertebrate rotifer *Brachionus manjavacas*. Rotifers are a promising model organism for life extension studies as they maintain a short, measurable lifespan while also having an accepted literature precedent related to aging. Rotifer lifespan was increased 9-14% by exposure to three of 200 screened red algal extracts. Bioassay guided fractionation led to semi-purified extracts composed primarily of lipids responsible for rotifer life extension. The life extending effects of these small molecule mixtures are not a result of their antioxidant capacity; instead they may activate pathways that slow the accumulation of cellular damage. An understanding of how these natural products interact with their molecular targets could lead to selective and efficient treatments for slowing aging and reducing age related diseases.

CHAPTER 1

INTRODUCTION

Background

Improving health and reducing the prevalence and severity of disease may be achieved by slowing the aging process and the accumulation of cellular damage. Biochemical changes associated with macromolecular damage, removal of DNA tags, shortening of telomeres, and alterations in the histone code that change genetic expression all cause a decrease of tissue and organ function resulting in aging.[1] The free radical hypothesis is one prominent explanation of age related changes in cellular activity[2] whereby reactive compounds are proposed to interact with macromolecules, such as DNA or lipids, generating mutations or carcinogens.[3] Free radicals often consist of reactive oxygen species (ROS) produced as chemical messengers or as metabolic by-products.[4,5] Cells contain proteins and protective pathways that can mitigate ROS and repair damage that has already occurred.[6] The controlled modulation of these pathways offers the basis for slowing the progression of aging.

Age related disorders that are often treatable or preventable include cancer, neurodegenerative disorders, cardiovascular disease, and type II diabetes.[7] Although many modern medical procedures slow or reverse tissue damage resulting from these disorders, preventative treatments have shown greater impacts on human health.[7] One such approach is to activate natural protective pathways which may lead to a decrease in age related illness and an increase in human healthspan.[8]

Limiting food intake without causing malnutrition can increase mean mammalian lifespan by up to 60% while decreasing rates of degenerative diseases.[8,9] However, effective dietary limitation has not gained general acceptance due to the strict dietary requirements involved.[8] In laboratory trials testing caloric restriction, aging has been slowed and lifespan increased in rotifers, flies, worms, and mice.[1,9] Small molecule drugs that mimic the effects of caloric restriction, without decreasing quality of life, could slow aging and increase healthspan.[10,11]

Past Approaches and Accomplishments

Several drug candidates aimed at mimicking the effects of caloric restriction have focused on eliminating ROS to slow accumulation of cellular damage.[3,12] However, this narrow focus has not yet yielded safe and effective treatments. One small molecule that extends mammalian lifespan is rapamycin, a macrocyclic polyketide derived natural product from bacteria .[1,13] Mice showed 9-14% increase in lifespan when treated with rapamycin due to changes in energy metabolism resulting from the inhibition of the mechanistic target of rapamycin complex 1 (mTOR1).[7] However, rapamycin also causes insulin resistance, increasing the risk for type II diabetes, due to increased blood glucose levels.[7] Rapamycin is approved for clinical use in preventing the rejection of organs after transplants, but at a dose one thirtieth to one sixtieth required for lifespan extension.[14] These discoveries illustrate that small molecules can extend mammalian lifespan, but a safe and efficacious molecule suitable for long term clinical use has not been identified.

Current Research Methods

In order to facilitate discovery of new mechanisms of action to slow aging, a phenotypic assay simultaneously incorporating multiple molecular targets is preferential to a targeted approach due to our limited understanding of aging. The use of a targeted approach is more useful to optimize small molecule based treatments once druggable molecular targets are identified. A whole animal screen measuring lifespan upon exposure to a diverse library of small molecules could lead to new therapeutics and the identification of biochemical pathways relevant to aging. Structurally diverse mixtures of small molecules function well in phenotypic assays, and for this reason a library of Fijian red algal extracts containing mixtures of natural products was selected for screening. Marine algae represent a rich source of structurally diverse small molecules including essential vitamins and minerals, polyunsaturated fatty acids, and antioxidants.[15-17] Red algae continue to be a source of natural product discovery with 42 new molecular structures identified in 2011.[18]

Phenotypic assay development requires use of a model organism complex enough to share many traits with humans while having a short generation time and life cycle amenable to medium- or high-throughput screening. This need has led to the use of *Saccharomyces cerevisiae, Caenorhabditis elegans, Drosophila melanogaster, Brachionus manjavacas, Danio rerio,* and *Mus musculus* as model organisms in aging assays.[8,19,20] Even though mice have the greatest genetic homology to humans (of the species listed above), their long lifespan and ethical considerations make them unsuitable for even medium-throughput screening. From this list, budding yeast (*S. cerevisiae*) is the least genetically similar to humans with substantial differences in reproduction which

is the phenotypic marker of yeast lifespan.[21] Additionally, yeast cannot serve as a model for neurological and muscular cells that do not divide, nor for the successful maintenance of cells that retain their functionality following differentiation.[22] As members of the Ecdysozoa superphylum of invertebrates, *C. elegans* and *D. melanogaster* are two common model organisms, but they have each undergone extensive gene loss since their common ancestor with humans.[19,23]

Brachionus manjavacas is a small, multicellular invertebrate animal of the Rotifera phylum, evolutionarily divergent from *C. elegans* and *D. melanogaster* with five main features favoring it for aging research. First, rotifers have a short lifespan of about two weeks.[23] Second, rotifers have become a well documented and accepted model to test aging hypotheses.[19,24-26] Third and fourth, they are able to reproduce asexually resulting in minimal genetic diversity within a medium-throughput assay utilizing many individuals, yet they can also reproduce sexually to produce stable diapausing eggs which can be stored for subsequent use.[24] Finally, a well-developed background of genetic resources including a partially sequenced genome and transcriptome[24] coupled with techniques for RNA interference to repress the expression of possible molecular targets[27] facilitates the identification of proteins and networks related to aging. When rotifers hatch, cellular division ceases and cells remain in a postmitotic state.[19] This does not allow all mechanisms of aging to be tested, but does represent a model for mammalian cells such as neurons and cardiomyocytes that undergo hypertrophy and not hyperplasia. The combined genetic resources and medium-throughput potential of rotifers make them strong candidates for aging-related phenotypic assays.

We screened 200 red algal extracts from approximately 34 genera for the discovery of molecules that extend rotifer lifespan. We tested whether life-extending extracts have a direct antioxidant effect that would enable them to protect cells from ROS, or if a more complex mechanism was involved. Mixtures of life-extending natural products were partially characterized using nuclear magnetic resonance spectroscopy and mass spectrometry. This represents a first step in generating potential therapeutics and small molecules that could be used as molecular probes to understand the pathways involved in aging.

CHAPTER 2

MATERIALS AND METHODS

Sample Collection and Identification

Red algal samples were collected offshore of the Fijian islands at depths of 2-20 m, between July 2006 and August 2010. The date and location of each collection was recorded along with a photograph, a formalin preserved sample, and a DNA voucher in ethanol. The collections were composed of at least 34 red algal genera (including six extracts from unknown red algal genera). Specimens were identified by the comparison of morphological traits to that of known algae, or occasionally through 18S rRNA sequencing. Extracts found to extend rotifer lifespan (*Acanthophora spicifera* (G-0548), *Jania acutilobum* (G-0062), and *Peyssonnelia* sp. (G-0565)) were pursued beyond extract screening. *Acanthophora spicifera* was collected near the Fijian island of Nacula (S16^{o} 55'43", W177^{o} 23'52"), *J. acutilobum* was collected near Viti Levu (S18^{o} 12'6", W177^{o} 39'39"), and *Peyssonelia* sp. was collected near Malolo (S17^{o} 16'24", W177^{o} 6'3").

Generation of Algal Extracts

Small organic molecules were extracted from freshly collected red algae (each sample measuring 20 mL by volumetric displacement) with successive exposure to methanol for 6-16 hours. Crude extracts were filtered to remove insoluble material and the methanol removed *in vacuo*, followed by vacuum liquid chromatography using 20 g of Diaion HP20ss resin. Each crude extract was adsorbed onto resin and washed with 50 mL deionized water to remove salts. Three extract fractions (hereafter, extracts) of

decreasing in polarity were eluted with (1) methanol/water 1:1, (2) methanol/water 4:1, and (3) 100% methanol. Solvents were removed *in vacuo* and extracts were dissolved in dimethyl sulfoxide (DMSO) at 20 mg/mL and stored in 96 well plates at -80 °C until screened in the rotifer life extension assay.

Screen of Algal Extracts for Rotifer Life Extension

The effects of 200 red algal extracts were each examined in the assay monitoring lifespan (Appendix A) of the model organism *B. manjavacas*, originally collected from the Azov Sea region in Russia.[19] The same assay design was used to test chromatographic fractions resulting from fractionation of positive hit extracts towards isolation of the compounds responsible for life extension. Rotifers were hatched from diapausing eggs for each experiment by incubation in 15 ppt artificial seawater and fed the alga *Tetraselmis suecica* ad libitum within 4 hours. Aging assays were conducted with individual rotifers grown in wells containing 200 µL of 15 ppt artificial seawater, 3.0 µg 5-fluro-2'-deoxyuridine (FUDR) to prevent reproduction,[19] , and 10^6 cells/mL *T. suecica*. Individual rotifers (n=12 each in its own well of a 96 well plate) were exposed to an algal extract at a concentration of 0, 2, or 10 µg/mL (delivered in 1 µL of DMSO). Rotifers were redosed after 7 days with 100 µL of 15 ppt artificial seawater, 1.2 µg FUDR, 10^6 cells/mL of *T. suecica*, and 0.5 µL DMSO containing 0, 0.2, or 1.0 µg algal extract. Rotifers were observed daily and scored as alive or dead, and experiments concluded after 15 days. Survivorship curves were constructed and analyzed by performing a two tailed logrank test comparing treatments with their unique corresponding controls at a threshold for significance level of α=0.05 using GraphPad Prism version 4.00 for

Windows. Extracts found to have a positive effect on rotifer lifespan were subjected to a repeated lifespan assay with 48 replicate individual rotifers and four extract concentrations of 1-20 µg/mL. If the results of the secondary assay also showed that the extract significantly extended rotifer lifespan, then the extract was considered a positive hit.

Antioxidant Efficacy Assay

A colorimetric assay allowed comparison of the antioxidant efficacy of algal extracts to that of the antioxidant 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox). The assay was conducted as described previously.[28] Briefly, an aqueous solution of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) (7.0 mM) and potassium persulfate (2.5 mM) was allowed to incubate in the dark for 12-14 hours. The resulting radical cation was diluted 50 fold with deionized water and pipetted into a 96 well plate. The ABTS assay consisted of a trolox positive control (7.1 µg/mL) that was compared to antioxidant effects of algal extracts (tested at 132 µg/mL) with six replicates. After 3 hours incubation, the absorbance of each well was measured at a wavelength of 630 nm using an automated plate reader (BioTek EL-800) with lower absorbance values corresponding to greater antioxidant capacity. Antioxidant capacities relative to trolox were calculated from the equation $\frac{control-extract}{control-trolox}$ with each value as absorbance at 630 nm and where "control" is the absorbance for a negative control in which no antioxidants were present. Using GraphPad Prism 4.00 a correlation statistical analysis was performed with a two tailed nonparametric Spearman test to determine if a relationship existed between antioxidant efficacy and lifespan extension.

Extract Fractionation

Positive hit red algal extracts were further fractionated using size exclusion or polarity based chromatography for those fractions that showed significant life extending effects (Figure 1). Size exclusion chromatography was performed using Sephadex LH-20 with methanol as mobile phase. Active fractions were further separated with polarity based flash chromatography using a 10 g Supelco ENVI-18 solid phase extraction column, eluting with aqueous methanol solutions of decreasing polarity (25-100% methanol). Chromatographic fractions were pooled based on common thin layer chromatography (TLC) characteristics, and each pooled fraction was tested in the rotifer life extension assay at an optimum equivalent concentration determined from HP20ss fractions. Life extending chromatographic fractions from the ENVI-18 separation were subjected to high performance liquid chromatography (HPLC) using a Waters 1525 binary pump system coupled to Waters 2996 diode array UV detection. Multiple rounds of HPLC separation to collect life extending small molecules were conducted using reversed phase 5 µm Grace Alltech C_{18} columns (10 x 250 mm and 4.6 x 250 mm) with gradient mobile phases of aqueous acetonitrile, methanol, and formic acid in mixtures of decreasing polarity. Normal phase HPLC was also utilized with 5 µm Agilent Technologies normal phase Zorbax columns (9.4 x 250 mm and 4.6 x 250 mm) using gradient mobile phases of hexanes and ethyl acetate in mixtures of increasing polarity.

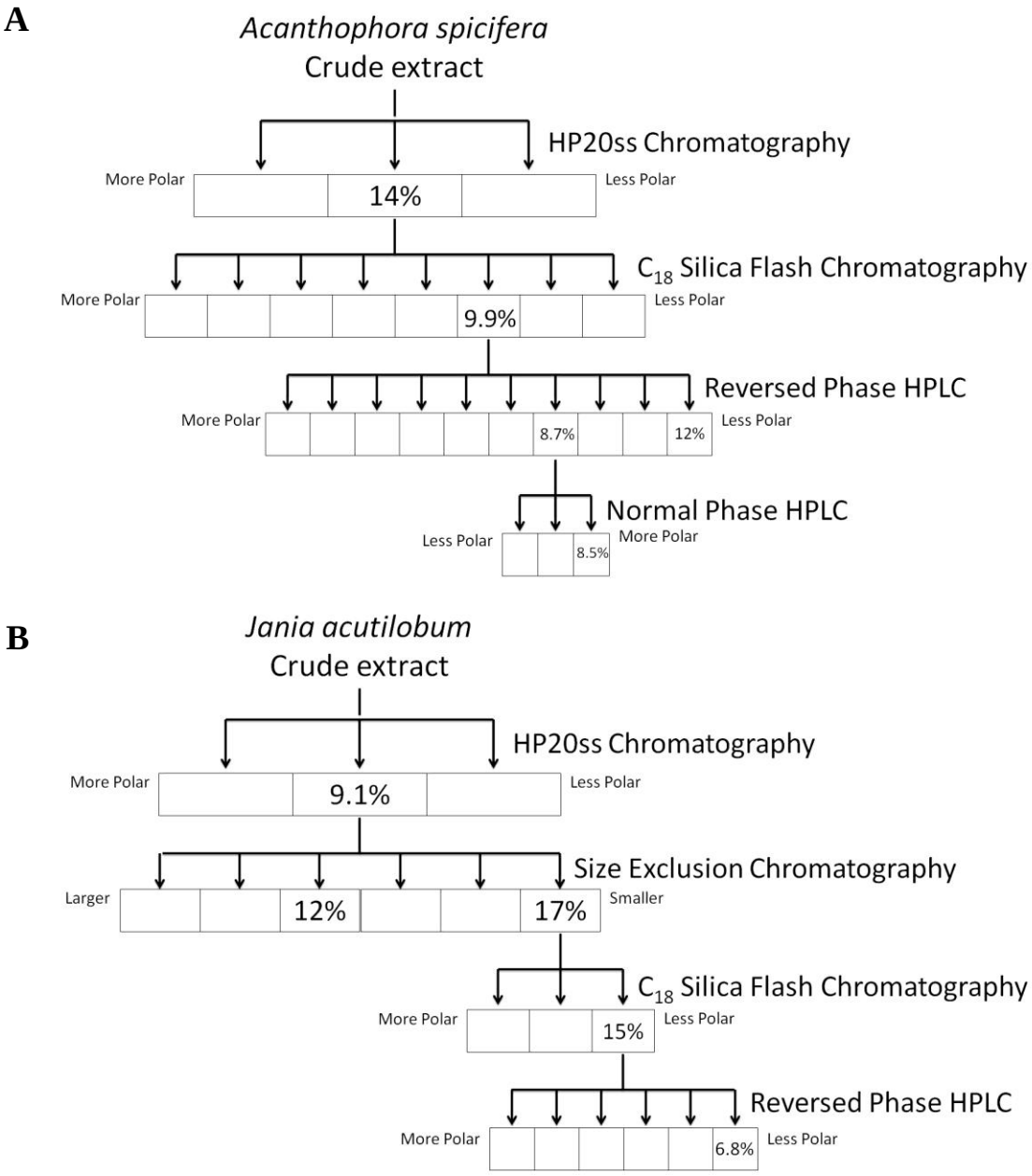


Figure 1: Bioassay guided fractionation procedure used for two red algal extracts, **(A)** *A. spicifera*, and **(B)** *J. acutilobum*, and with significant lifespan extension as percent of total lifespan noted for active fractions.

Spectroscopic and Spectrometric Analysis

Chromatographic fractions from positive hit red algal extracts were characterized using nuclear magnetic resonance (NMR) spectroscopy. NMR spectra were collected using a Bruker DRX-500 instrument with a 5 mm broadband or inverse detection probe for ^{1}H experiments with deuterated chloroform or DMSO supplied by Cambridge Isotope Laboratories. During the bioassay guided fractionation process, algal extract fractions were also analyzed by liquid chromatography mass spectrometer (LC-MS) with a Waters 2695 HPLC pump coupled to a Waters 2996 diode-array UV detector and Micromass ZQ 2000 mass spectrometer with electrospray ionization. LC-MS separation was accomplished using of a 3 µm Grace Alltech C_{18} silica reversed phase column (2.1 x 150 mm) and water/acetonitrile/acetic acid or water/methanol/acetic acid mobile phases. LC-MS was also used to verify the completion of the esterification reaction (below).

Esterification of Fatty Acids

The facile analysis of fatty acid methyl esters (FAMEs) by gas chromatography/mass spectrometry (GC-MS) makes esterification of fatty acid derivatives a useful tool for identification by comparison with known standards. Carboxylic acids were esterified (and esters were transesterified) for characterization of life extending fatty acid derivatives within chromatographic fractions of positive hit extracts. Esterification was conducted as previously described.[29] Briefly, concentrated hydrochloric acid (HCl) was diluted to 8% (w/v) with methanol and water (85:15). Each extract fraction to be esterified (1.2 mg) was dissolved in a solution of 0.2 mL toluene and 1.5 mL methanol followed by the addition of 0.3 mL of the 8% HCl solution. This

yielded a final HCl concentration of 1.2% (w/v) that was incubated at 45 ^{o}C for 14 hours. Following dilution with deionized water (1.0 mL), FAMEs were extracted from the product mixture with hexanes (1.0 mL) and partially purified by normal phase HPLC (see above). FAMEs were characterized using a HP 6890 gas chromatograph coupled to a Waters AutoSpec mass spectrometer. Fragmentation patterns were compared to the NIST/EPA/NIH mass spectral library for electrospray ionization.

Quantification of Fatty Acid Derivatives Using HPLC-ELSD

Quantification of extract mass required for trolox antioxidant equivalent capacity was accomplished using the comparison of the internal standard peak area of *trans*-stilbene to *J. acutilobum* and *A. spicifera* extract total peak areas (Figure 2). Because masses were too low for quantification using an analytical balance, quantification of extract fractions was accomplished by HPLC (see above) coupled with an Alltech 800 evaporative light scattering detector (ELSD) as previously described[30]. An Agilent Technologies 5 µm Zorbax normal phase silica column, (4.6 x 250 mm) separated fatty acid derivatives within each extract fraction with a linear gradient mobile phase of 100% hexanes to 100% ethyl acetate over 30 minutes (with flow rate of 1.0 mL/min). The ELSD drift tube temperature operated at 21 ^{o}C with a nitrogen pressure of 2.11 bar. An internal standard of *trans*-stilbene (50-70 µg) was used to calculate the total mass of fatty acid derivatives within each extract fraction by integration of ELSD peaks.

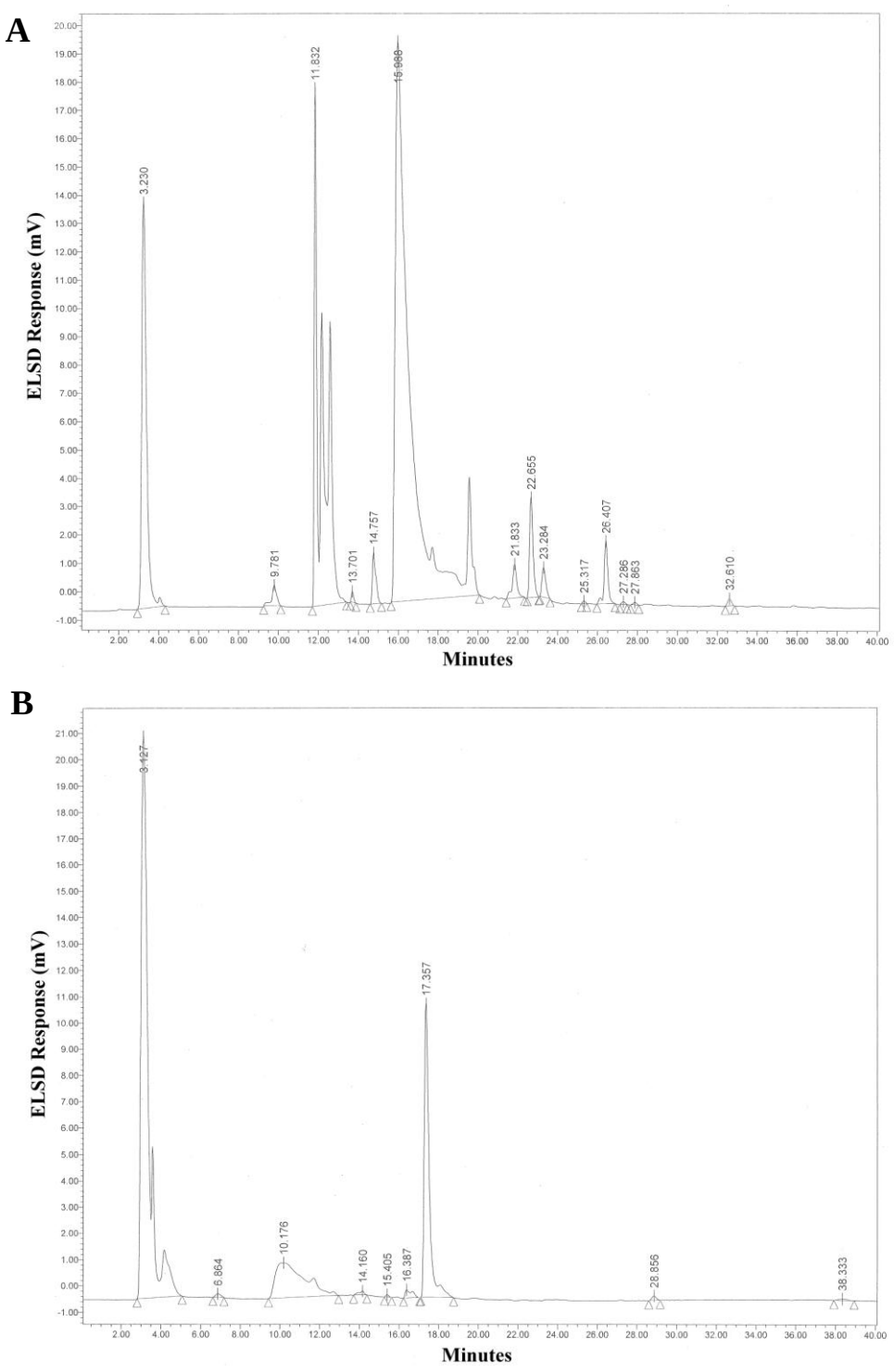


Figure 2: HPLC chromatograms of partially purified compounds from red algae detected by evaporative light scattering detection (ELSD). **(A)** *Acanthophora spicifera* lipid mixture, spiked with 50 µg *trans*-stilbene (eluting at 3.5 minutes). **(B)** *Jania acutilobum* mixture spiked with 70 µg of *trans*-stilbene (eluting at 3.5 minutes).

CHAPTER 3

RESULTS

Screening Algal Extracts

Rotifer lifespan was extended by eight of the 200 red algal extracts tested at doses of 2 or 10 µg/mL (each dose n=12; p=0.01-0.05 as determined by a logrank test) (Figure 3A and B). When the eight life extending extracts were retested with 48 replicates each at four concentrations of 1-20 µg/mL, only three extracts were found to reproducibly cause rotifer life extension. Extracts of *A. spicifera, J. acutilobum,* and *Peyssonnelia* sp., all members of the red algal class Florideophyceae, were found to each extend mean and maximum rotifer lifespan. In the repeated assays, mean rotifer lifespan was extended by 14% for 5 µg/mL of *A. spicifera* extract (p=0.034), 9.1% for 5 µg/mL of *J. acutilobum* extract (p=0.027), and 8.5% for 2 µg/mL of *Peyssonnelia* sp. extract (p=0.048) (Figure 4). In some cases, exposure to concentrations of algal extracts above those extending lifespan resulted in toxic effects on rotifers (data not shown).

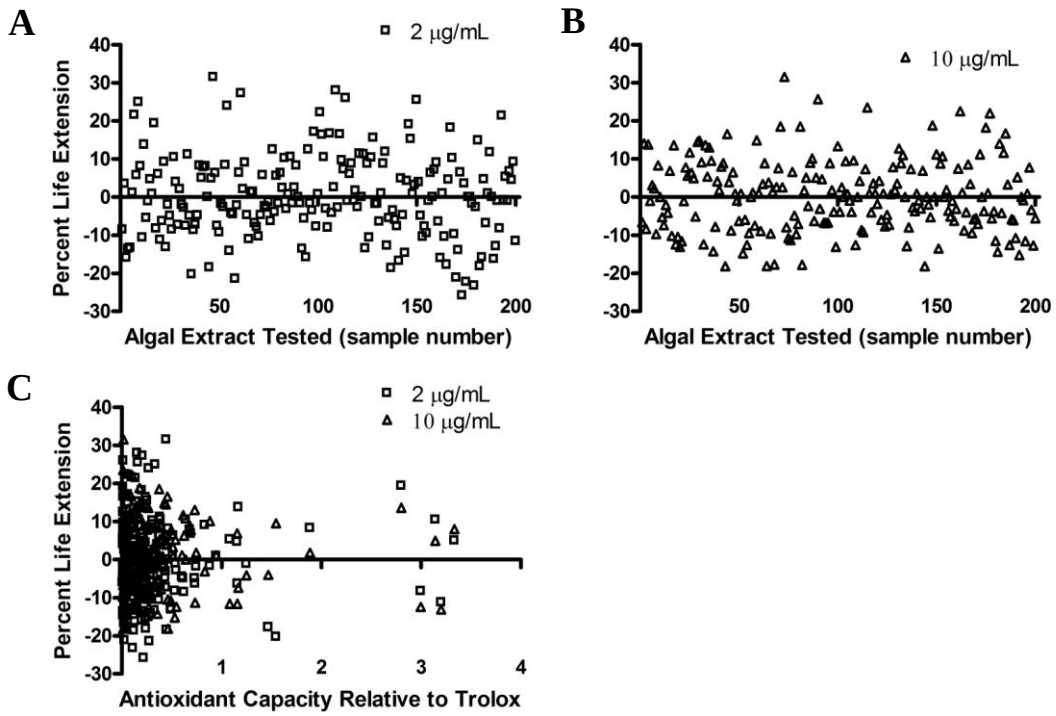


Figure 3: Effect on rotifer lifespan (n=12 each) of exposure to 200 different red algal extracts at **(A)** 2 μg/mL and, **(B)** 10 μg/mL. (**C)** No relationship was apparent between rotifer life extension and antioxidant capacity for either the 2 or 10 μg/mL dose of 200 red algal extracts ($p=0.73$ and 0.67 respectively with $r^2<0.0001$ for both).

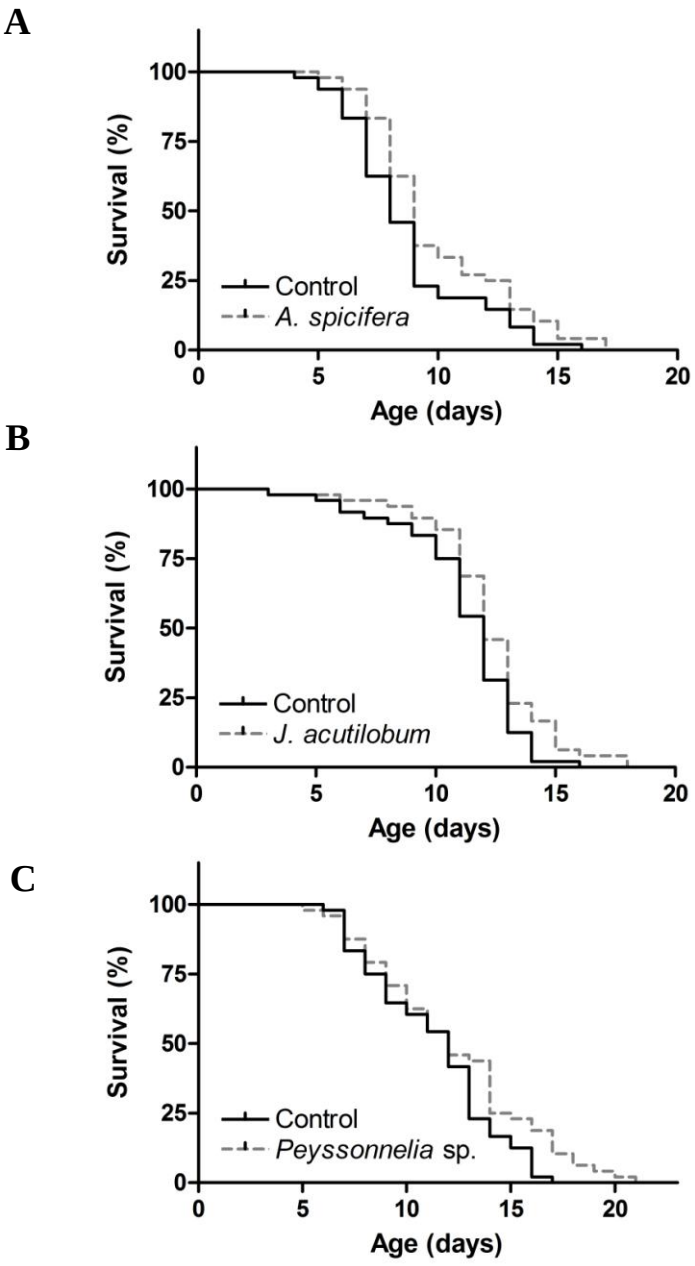


Figure 4: Three red algal extracts extended rotifer lifespan as determined by a two tailed logrank test (n=48 each). Effect of **(A)** *Acanthophora spicifera* extract at 5 µg/mL (rotifer lifespan extension 14%; p=0.034), **(B)** *Jania acutilobum* extract at 5 µg/mL (rotifer lifespan extension 9.1%; p=0.027), and **(C)** *Peyssonnelia* sp. extract at 2 µg/mL (rotifer lifespan extension 8.5%; p=0.048).

Relationship between Life Extension and Antioxidant Efficacy

No significant positive or negative correlation was observed between rotifer lifespan and antioxidant capacity for the 200 red algal extracts at 2 µg/mL (r^2<0.0001, p=0.73) or 10 µg/mL (r^2<0.0001, p=0.67) (Figure 3C). Each of the three confirmed life extending algal extracts had antioxidant capacities well below that of trolox, a known antioxidant even when tested at almost 20 times the concentration of trolox (Figure 5). Partially purified lipids with life extending properties from *A. spicifera* and *J. acutilobum* did not exhibit substantial antioxidant effects either: each small molecule mixture demonstrated antioxidant capacity of less than 1% of trolox, once differential dilution was taken into account (Figure 5).

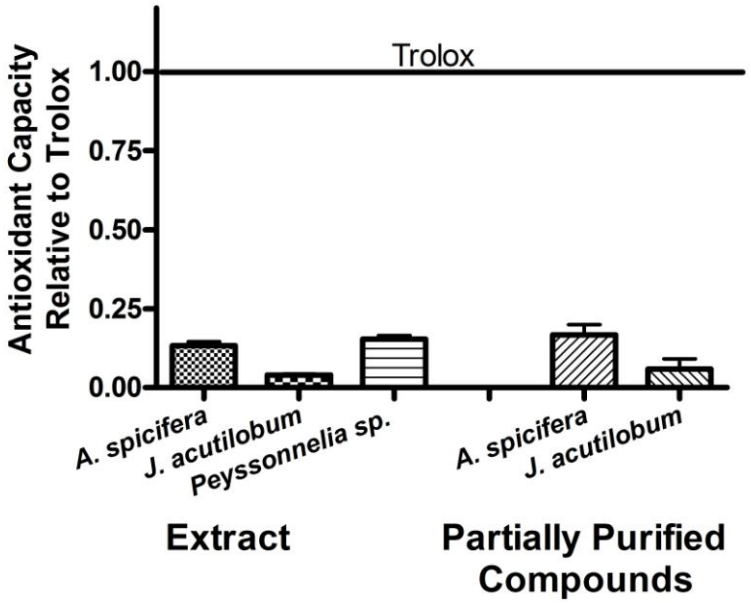


Figure 5: Life extending red algal **(A)** extracts and **(B)** partially purified compounds exhibited antioxidant capacities considerably less than trolox, (in this experiment, the concentration of trolox was approximately 1/20th that of algal extracts or compounds).

Bioassay Guided Fractionation of Algal Extracts

When the life extending extract of *A. spicifera* was subjected to flash chromatography on reversed phased silica gel, one active fraction extended rotifer lifespan by 9.9% (p=0.031; Figure 1A). Fractionation with reversed phase HPLC yielded two active fractions extending rotifer lifespan, by 8.7% (p=0.028) and 12% (p=0.026). While further separation by normal phase HPLC of the latter fraction resulted in decomposition, separation of the former fraction resulted in one fraction extending lifespan by 8.5% (p=0.038). This fraction was composed of several fatty acid derived molecules, based upon TLC, NMR, and MS analysis (see below). Unfortunately, additional HPLC fractionation resulted in decomposition of life extending compounds.

When the life extending *J. acutilobum* extract (Figure 1B) was fractionated by size exclusion chromatography, two fractions extended lifespan, by 12% and 17% (p=0.018 and p=0.003, respectively). Further separation by reversed phase flash chromatography of the former fraction resulted in decomposition, while separation of the later fraction resulted in one fraction exhibiting a 15% increase in rotifer lifespan (p=0.034). Reversed phase HPLC separation of the active fraction served to remove additional impurities while retaining rotifer life extension of 6.8% (p=0.048), but failed to result in a pure compound.

When the *Peyssonnelia* sp. extract which extended rotifer lifespan by 8.5% (p=0.048) was fractionated by size exclusion chromatography, activity was lost, indicating lack of stability of the life extending compound(s).

Characterization of Life Extending Small Molecules

LC-MS analysis of life extending extract fractions from *A. spicifera* and *J. acutilobum*, indicated that each contained approximately 10-30 compounds with molecular weights in the range of 250-400 Da. ^{1}H NMR spectral analysis of the partially purified active fraction from *A. spicifera* spectra revealed prominent concentrations of long chain alkyl groups indicative of products of fatty acid biosynthesis (Figure 6A). Fatty acid derived molecules were evident by the presence of methyl triplets at 0.9-1.0 ppm, methylene protons with and without adjacent carbonyl groups from 1.2-2.5 ppm, as well as several overlapping olefinic protons at 5.4 ppm (Figure 6A). The *J. acutilobum* fraction ^{1}H NMR spectra revealed a mixture of small molecules with the presence of one or more alkenes evident from peaks at 5.5-6.5 ppm (Figure 6B). The mixture does not show evidence for the presence of fatty acids due to the low abundance of methylene protons having peaks at 1.2-2.5 ppm. There is evidence for a methoxy functional group at 3.4 ppm, and possibly a carbonyl with a methyl singlet at 2.1 ppm (Figure 6B). Due to the low fraction concentration and impurities it is not possible to fully characterize the molecular structure.

Acanthophora spicifera ^{1}H NMR spectra showed the presence of fatty acids, but it was not possible to characterize individual fatty acids within this mixture, nor to purify them without loss of activity. Thus, methyl esters were prepared from life extending mixtures of *A. spicifera* lipids, resulting in multiple methylated products which could be analyzed by GC-MS. Fragmentation patterns generated from GC-MS analysis enabled identification of eicosanoic, octadecanoic, and hexadecanoic acid methyl esters and several unsaturated derivatives to a lesser degree of certainty (Appendix B).

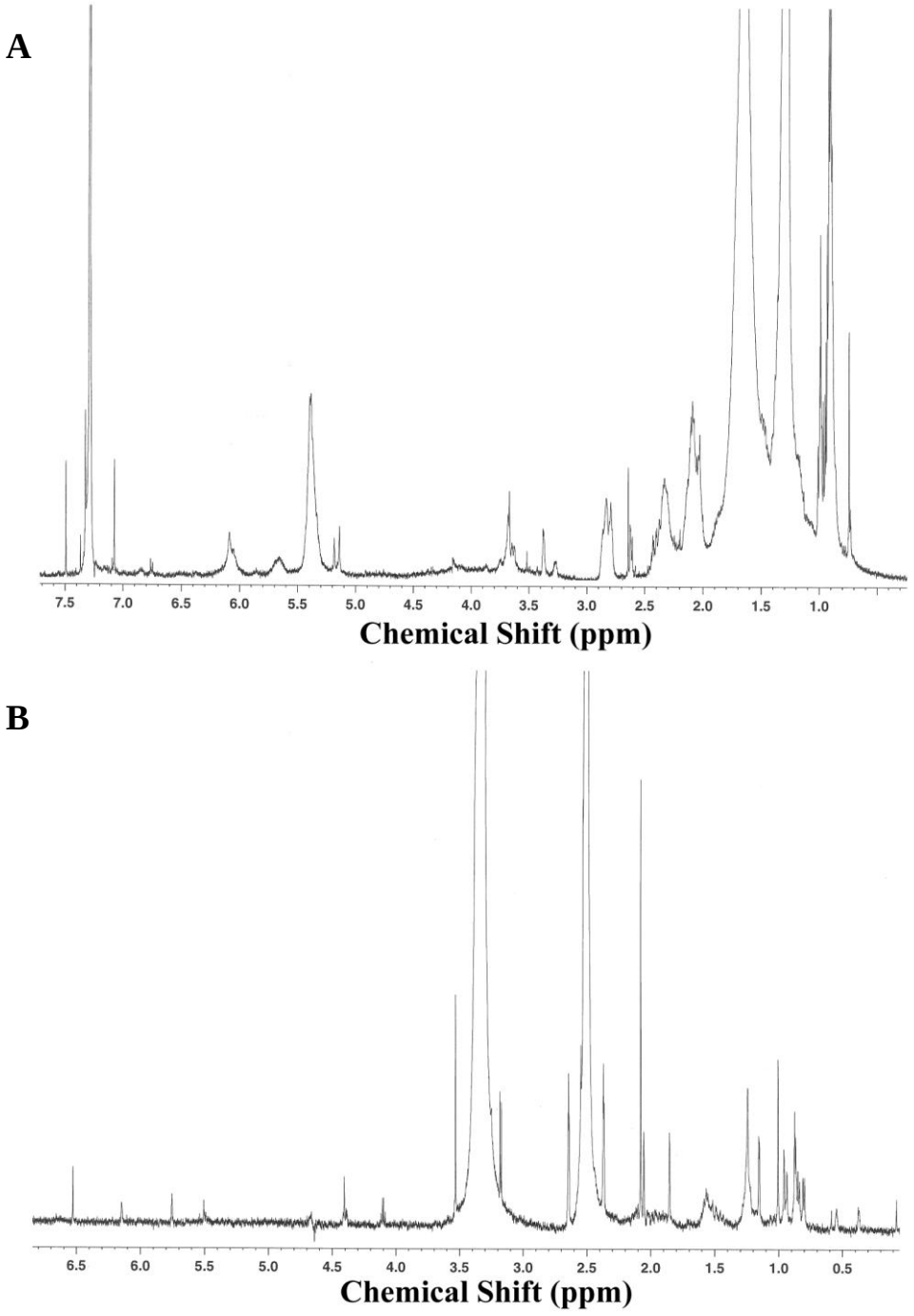


Figure 6: ^{1}H NMR spectra of life extending red algal fractions. **(A)** Lipids from *A. spicifera* (in $CDCl_3$), and **(B)** partially purified mixture from *J. acutilobum* (in DMSO-d_6).

Compound quantification achieved by HPLC-ELSD analysis in comparison with an internal standard (*trans*-stilbene) resulted in a determination that the *A. spicifera* life extending fraction contained 320 µg fatty acids and fatty acid derivatives. The injected mass allowed for the determination that 2.2 mg (640 µg remained after assays) had been isolated from 12.2 g, representing a total of 0.018% of algal dry mass (Figure 6). For the mixture of life extending small molecules from *J. acutilobum*, HPLC-ELSD analysis resulted in quantification of 43 µg from a 1.9 mg fraction (344 µg remained after assays) which represented 0.053% of algal dry mass.

Antioxidant Efficacy of Life Extending Small Molecule Mixtures

Analysis of the antioxidant efficacy revealed that the life extending mixture of lipids from *A. spicifera* exhibited less than 1.0% of the antioxidant capacity of trolox (i.e., 111 µg of *A. spicifera* lipid mixture exhibited similar antioxidant effect as 1.0 µg trolox) (Figure 5). Life extending small molecules from *J. acutilobum* exhibited less antioxidant capacity, approximately 0.32% that of trolox (i.e., 312 µg of *J. acutilobum* lipid mixture was equivalent in antioxidant effect to 1.0 µg trolox). Thus, it appears that antioxidant effects are very modest for these mixtures of algal small molecules which extend rotifer lifespan.

CHAPTER 4

DISCUSSION AND CONCLUSION

A small number of red algal extracts (three of 200) were found to reproducibly extend the lifespan of the model invertebrate *B. manjavacas* by 8.5-14% (Figs. 3-4). These life extending effects (Figure 4) are similar in magnitude to previous rotifer lifespan extension studies that resulted in a maximum mean lifespan increase of 16-17%.[19,31] In contrast, caloric restriction has been shown to extend rotifer lifespan by 50%.[6] Each of these findings are dwarfed by the 300% lifespan increase for rotifers treated with indolepropionamide,[26] but no rotifer lifespan extension was observed when tested under slightly different circumstances in a later study.[19] These previous studies have focused on antioxidants as target molecules, but our results indicate that protection against oxidative stress is not necessarily the mechanism by which small molecules extend life (Figs. 3C, 5). Studies focusing on *in silico* screening of small molecules have predicted life extending drug candidates that are not antioxidants.[32]

Due to decomposition during purification, the molecular structures of the small molecules responsible for rotifer life extension were not fully elucidated. However, NMR and mass spectral analyses indicated that the red alga *A. spicifera* contained life extending lipids accounting for 80-90% of the active fraction. This lipid mixture was partially characterized by GC-MS as the corresponding fatty acid methyl esters, identifying components in the algal extract including eicosanoic, octadecanoic, and hexadecanoic acids as well as a number of unidentified unsaturated lipids. It is unlikely that these lipids act as simply as supplements to the rotifer diet, thereby extending life,

since in the life extension assay rotifers had access to an abundant algal food (*Tetraselmis*) known to be rich in fatty acids and other essential nutrients.[33] Fatty acids, such as hexadecanoic and octadecanoic acid have been shown to be a pro-oxidant,[34] which further suggests antioxidant effects are unlikely to explain the effectiveness of these lipid mixtures.

It is plausible that the life extending red algal lipids acted as signaling molecules in pathways related to aging.[35-37] Fatty acids regulate transcription of genetic pathways related to energy metabolism through binding with peroxisome proliferator-activated receptors.[38] Activation of transcription factors associated with critical energy metabolism pathways has been the underlying mechanism by which caloric restriction and rapamycin are hypothesized to extend lifespan.[9,39] . Fatty acids affecting genetic pathways include 10-hydroxy-2-decanoic acid which is present in high concentrations in lifespan extending royal jelly responsible for queen bee development.[36] Fatty acids may increase lifespan through the activation of transcription factors resulting from a minor toxic effect,[40] or as a ligand with targeted binding.[41] Energy metabolism in the mitochondria impacts aging through the production of ROS, but complex networks of proteins and signaling molecules exist to neutralize ROS and repair damage.[42,43] Controlling or manipulating mitochondrial networks is one focus for reducing cellular damage,[42,44] and represents a putative mechanism of action for the lipid mixtures in this study.

The use of a library of small molecule drugs to examine effects on lifespan will allow for the discovery of new mechanisms of action related to aging without requiring *a priori* knowledge of, or bias towards, known molecular targets.[45] For drug discovery, phenotypic approaches have generally been more effective than target based screening

for first-in-class molecules.[45] Performing survivorship analysis for the full lifespan of a model organism is time consuming, but presents the opportunity to identify novel pathways for preventative treatments. Additionally, the critical involvement of mitochondria in metabolism, aging, and disease indicate that pathways activated in rotifers would also be affected in humans and other vertebrates due to the highly conserved targets of the mitochondrial genome. [46] In the current study, we did not directly identify pathways affected by the life extending small molecules of red algae. However, in the process of screening many small molecules extracted from red algae, we found that antioxidant properties did not account for any of our life extending hits (Figs. 3C, 5). With the 200 red algal extracts studied, no significant correlation was observed between antioxidant efficacy and lifespan extension (Figure 3C), but these results do not refute earlier data that demonstrated oxidative damage is a contributor to aging.[2,19] With a lack of observed antioxidant effects among the life extending algal extracts or small molecule mixtures, our results indicate that efficacious small molecules do not neutralize ROS through direct reactions, and likely activate protective pathways.[1,47]

Research towards slowing the effects of aging has allowed for a better understanding of the aging process, but the goal to identify bioactive small molecules remains. The current work indicates that natural mixtures of lipids from marine algae exhibit useful life extending properties, with the scope of drug candidates expanded to include molecules that appear not to act as antioxidants. The use of macroalgae as a source of structurally diverse natural products has shown to be effective, and further characterization of life extending molecules should continue.

APPENDIX A

EXTRACT INFORMATION AND ASSAY RESULTS

Extract Genus	Extract Species	Sample ID#	Percent Life Extension of 2 µg/mL Dose	P Value of 2 µg/mL Dose	Percent Life Extension of 10 µg/mL Dose	P Value of 10 µg/mL Dose	AO Efficacy
GALAXAURA	MARGINATA	G-0020-1	-8.5%	0.63	-6.6%	0.54	0.089
GALAXAURA	MARGINATA	G-0020-2	3.5%	0.68	14.1%	0.36	0.143
GALAXAURA	MARGINATA	G-0020-3	-15.9%	0.20	-8.4%	0.85	0.202
CALLOPHYCUS	SERRATUS	G-0021-1	-13.5%	0.23	13.8%	0.24	0.203
CALLOPHYCUS	SERRATUS	G-0021-2	-13.3%	0.24	-1.0%	0.92	0.328
CALLOPHYCUS	SERRATUS	G-0021-3	1.2%	0.78	3.1%	0.76	0.342
PEYSSONNELIA	INAMOENA	G-0044-1	21.6%	0.13	2.3%	0.91	0.099
PEYSSONNELIA	INAMOENA	G-0044-2	5.8%	0.72	-9.7%	0.78	0.216
PEYSSONNELIA	INAMOENA	G-0044-3	25.0%	0.11	8.3%	0.47	0.335
NEURYMENIA	FRAXINIFOLIA	G-0053-1	8.1%	0.64	0.0%	0.88	0.270
NEURYMENIA	FRAXINIFOLIA	G-0053-2	-10.6%	0.42	-5.3%	0.77	0.373
NEURYMENIA	FRAXINIFOLIA	G-0053-3	13.8%	0.20	-7.4%	0.84	1.168
CHEILOSPORUM	ACUTILOBUM	G-0062-1	4.6%	0.12	-3.3%	0.53	0.033
CHEILOSPORUM	ACUTILOBUM	G-0062-2	25.8%	0.03	1.2%	0.11	0.041
CHEILOSPORUM	ACUTILOBUM	G-0062-3	14.7%	0.41	8.6%	0.32	0.126
PHACELOCARPUS	NEURYMENIOIDES	G-0068-1	-5.4%	0.59	-2.1%	0.67	0.290
PHACELOCARPUS	NEURYMENIOIDES	G-0068-2	-1.1%	0.86	-4.1%	0.74	1.249
PHACELOCARPUS	NEURYMENIOIDES	G-0068-3	4.7%	0.65	6.9%	0.68	1.157
PHACELOCARPUS	NEURYMENIOIDES	G-0074-1	0.9%	0.67	-10.2%	0.60	0.270
PHACELOCARPUS	NEURYMENIOIDES	G-0074-2	19.4%	0.25	13.6%	0.59	2.801
PHACELOCARPUS	NEURYMENIOIDES	G-0074-3	-8.3%	0.31	-12.4%	0.29	2.997
PEYSSONNELIA		G-0138-1	6.0%	0.66	-10.5%	0.31	0.461
PEYSSONNELIA		G-0138-2	-11.2%	0.25	-13.1%	0.32	3.200
PEYSSONNELIA		G-0138-3	-6.3%	0.92	-11.6%	0.30	1.160
CALLOPHYCUS	SERRATUS	G-0171-1	9.3%	0.55	-1.1%	0.87	0.305
CALLOPHYCUS	SERRATUS	G-0171-2	-13.1%	0.13	7.8%	0.74	0.495
CALLOPHYCUS	SERRATUS	G-0171-3	-2.0%	0.71	5.6%	0.65	0.233
PHACELOCARPUS	NEURYMENIOIDES	G-0192-1	-4.9%	0.62	11.7%	0.39	0.614
PHACELOCARPUS	NEURYMENIOIDES	G-0192-2	-8.5%	0.70	6.3%	0.49	0.624
PHACELOCARPUS	NEURYMENIOIDES	G-0192-3	10.5%	0.56	4.9%	0.46	3.144
AMPHIROA		G-0202-1	4.0%	0.82	-5.1%	0.65	0.353
AMPHIROA		G-0202-2	-7.3%	0.63	14.5%	0.19	0.441
AMPHIROA		G-0202-3	2.1%	0.71	14.8%	0.13	0.182
PORTIERIA		G-0205-1	-2.0%	0.62	9.1%	0.73	0.352
PORTIERIA		G-0205-2	-2.8%	0.89	-12.4%	0.49	0.545
PORTIERIA		G-0205-3	-7.5%	0.19	13.6%	0.21	0.265
NEURYMENIA	FRAXINIFOLIA	G-0206-1	11.2%	0.38	5.4%	0.22	0.381
NEURYMENIA	FRAXINIFOLIA	G-0206-2	-4.9%	0.50	13.0%	0.18	0.730
NEURYMENIA	FRAXINIFOLIA	G-0206-3	-20.3%	0.02	9.5%	0.59	1.545
AMPHIROA		G-0207-1	-8.6%	0.45	-14.3%	0.09	0.356
AMPHIROA		G-0207-2	-7.2%	0.64	-7.8%	0.84	0.389
AMPHIROA		G-0207-3	-4.8%	0.79	4.1%	0.65	0.434
PHACELOCARPUS		G-0216-2	8.3%	0.74	1.8%	0.89	1.886
PHACELOCARPUS		G-0216-3	5.0%	0.65	8.0%	0.68	3.334
ACTINOTRICHIA		G-0280-2	7.9%	0.59	8.9%	0.43	0.682
ACTINOTRICHIA		G-0280-3	8.1%	0.50	-18.2%	0.08	0.463
AMPHIROA		G-0282-2	0.0%	0.94	16.5%	0.15	0.456
AMPHIROA		G-0282-3	-18.4%	0.06	3.9%	0.81	0.451
TRICLEOCARPA		G-0309-1	4.9%	0.61	-8.8%	0.38	0.422
TRICLEOCARPA		G-0309-2	31.5%	0.06	6.5%	0.25	0.444
TRICLEOCARPA		G-0309-3	6.3%	0.70	0.9%	0.92	0.515
MESOPHYLLUM		G-0318-2	-7.5%	0.84	-0.9%	0.52	0.186
MESOPHYLLUM		G-0318-3	-9.2%	0.24	-6.3%	0.29	0.186
MESOPHYLLUM		G-0319-2	-1.8%	0.58	0.9%	0.88	0.410
MESOPHYLLUM		G-0319-3	-2.6%	0.56	-14.8%	0.01	0.256
AMPHIROA		G-0320-2	8.4%	0.62	-6.0%	0.77	0.264
AMPHIROA		G-0320-3	24.0%	0.27	-12.6%	0.41	0.270
DUDRESNAYA		G-0340-2	-14.1%	0.16	-9.5%	0.28	0.291
DUDRESNAYA		G-0340-3	-4.1%	0.86	0.0%	0.58	0.289
ACROSYMPHYTON	TAYLORII	G-0341-1	-4.5%	0.54	1.1%	0.86	0.603
ACROSYMPHYTON	TAYLORII	G-0341-2	-21.4%	0.02	-7.5%	0.30	0.276
ACROSYMPHYTON	TAYLORII	G-0341-3	-2.2%	0.73	14.9%	0.13	0.425
POROLITHON	GARDINERI	G-0361-2	6.4%	0.43	1.0%	0.77	0.380
POROLITHON	GARDINERI	G-0361-3	27.3%	0.05	-8.9%	0.40	0.205
ASPARAGOPSIS-PR	TAXIFORMIS	G-0372A-2	2.1%	0.77	3.4%	0.74	0.213
ASPARAGOPSIS-PR	TAXIFORMIS	G-0372A-3	9.1%	0.40	8.9%	0.29	0.034

Extract Genus	Extract Species	Sample ID#	Percent Life Extension of 2 µg/mL Dose	P Value of 2 µg/mL Dose	Percent Life Extension of 10 µg/mL Dose	P Value of 10 µg/mL Dose	AO Efficacy
AMPHIROA	CRASSA	G-0374-2	-4.7%	0.67	-18.1%	0.11	0.036
AMPHIROA	CRASSA	G-0374-3	-11.0%	0.16	3.8%	0.91	0.026
JANIA-PR		G-0382-2	1.3%	0.41	-9.6%	0.46	0.048
JANIA-PR		G-0382-3	1.4%	0.94	1.4%	0.72	0.035
AMPHIROA		G-0471-1	-6.8%	0.92	-17.7%	0.13	0.050
AMPHIROA		G-0471-2	-7.8%	0.24	2.6%	0.97	0.046
AMPHIROA		G-0471-3	-10.3%	0.14	7.6%	0.54	0.030
DASYPHILA	PLUMARIOIDES	G-0487-1	-4.7%	0.67	18.5%	0.27	0.017
DASYPHILA	PLUMARIOIDES	G-0487-2	5.8%	0.37	2.5%	0.69	0.036
DASYPHILA	PLUMARIOIDES	G-0487-3	-2.4%	0.88	31.5%	0.11	0.017
PEYSSONNELIA		G-0495-1	-2.7%	0.72	-7.9%	0.75	0.018
PEYSSONNELIA		G-0495-2	-1.3%	0.96	-10.8%	0.32	0.070
PEYSSONNELIA		G-0495-3	-6.3%	0.78	-11.3%	0.16	0.735
GIBSMITHIA	HAWAIIENSIS	G-0506-1	12.5%	0.32	6.6%	0.20	0.014
GIBSMITHIA	HAWAIIENSIS	G-0506-2	-3.9%	0.65	-4.9%	0.32	0.015
GIBSMITHIA	HAWAIIENSIS	G-0506-3	5.6%	0.52	-9.8%	0.34	0.011
AMPHIROA		G-0539-1	-1.6%	0.16	-7.1%	0.21	0.336
AMPHIROA		G-0539-2	6.3%	0.41	18.5%	0.77	0.374
AMPHIROA		G-0539-3	2.4%	0.22	-17.8%	0.15	0.431
ASTEROMENIA	PELTATA	G-0544-1	10.3%	0.21	1.7%	0.93	0.305
ASTEROMENIA	PELTATA	G-0544-2	-3.1%	0.68	5.1%	0.66	0.438
ASTEROMENIA	PELTATA	G-0544-3	0.8%	0.63	-2.4%	0.47	0.313
NEOGONIOLITHON	FRUTESCENS	G-0546-1	10.6%	0.12	9.0%	0.25	0.388
NEOGONIOLITHON	FRUTESCENS	G-0546-2	-1.6%	0.38	10.1%	0.12	0.884
NEOGONIOLITHON	FRUTESCENS	G-0546-3	2.5%	0.72	-6.3%	0.75	0.331
ACANTHOPHORA-PR	SPICIFERA	G-0548-1	8.3%	0.77	5.1%	0.63	0.018
ACANTHOPHORA-PR	SPICIFERA	G-0548-2	0.0%	0.79	25.7%	0.02	0.133
ACANTHOPHORA-PR	SPICIFERA	G-0548-3	-1.6%	0.83	-3.1%	0.91	0.213
HYPNEA		G-0549A-1	-13.5%	0.35	4.7%	0.55	0.012
HYPNEA		G-0549A-2	-5.6%	0.63	-6.7%	0.22	0.014
HYPNEA		G-0549A-3	-15.8%	0.09	-6.8%	0.36	0.031
HALYMENIA		G-0558-1	12.5%	0.15	-6.7%	0.92	0.012
HALYMENIA		G-0558-2	-1.6%	0.94	8.9%	0.49	0.014
HALYMENIA		G-0558-3	-3.5%	0.54	1.8%	0.44	0.085
PREDAEA		G-0560-1	17.1%	0.11	-3.9%	0.70	0.062
PREDAEA		G-0560-2	-7.7%	0.59	-13.1%	0.20	0.069
PREDAEA		G-0560-3	0.8%	0.95	13.3%	0.08	0.063
CORYNOCYSTIS	PROSTRATA	G-0564-1	22.3%	0.01	-3.9%	0.93	0.063
CORYNOCYSTIS	PROSTRATA	G-0564-2	16.4%	0.05	2.5%	0.56	0.240
CORYNOCYSTIS	PROSTRATA	G-0564-3	10.5%	0.17	0.0%	0.62	0.642
PEYSSONNELIA		G-0565-1	-3.0%	0.56	9.4%	0.98	0.031
PEYSSONNELIA		G-0565-2	-8.0%	0.11	0.8%	0.52	0.086
PEYSSONNELIA		G-0565-3	16.7%	0.22	-0.9%	0.45	0.036
PEYSSONNELIA		G-0565A-1	-4.1%	0.84	-3.9%	0.33	0.040
PEYSSONNELIA		G-0565A-2	2.6%	0.92	9.6%	0.22	0.023
PEYSSONNELIA		G-0565A-3	28.0%	0.05	-12.6%	0.46	0.153
GIBSMITHIA	HAWAIIENSIS	G-0568-1	-2.7%	0.63	0.0%	0.74	0.013
GIBSMITHIA	HAWAIIENSIS	G-0568-2	16.5%	0.25	4.2%	0.29	0.015
GIBSMITHIA	HAWAIIENSIS	G-0568-3	9.7%	0.39	-8.7%	0.39	0.015
HALYMENIA	DILATATA	G-0570-1	7.3%	0.22	-9.2%	0.41	0.015
HALYMENIA	DILATATA	G-0570-2	26.0%	0.02	7.3%	0.38	0.015
HALYMENIA	DILATATA	G-0570-3	-1.0%	0.79	23.5%	0.01	0.013
HALICHRYSIS	COALESCENS	G-0572-1	6.1%	0.30	0.9%	0.96	0.018
HALICHRYSIS	COALESCENS	G-0572-2	8.8%	0.60	-4.0%	0.96	0.049
HALICHRYSIS	COALESCENS	G-0572-3	-0.9%	0.96	3.6%	0.80	0.050
NEOGONIOLITHON	LACCADIVICUM	G-0574-1	1.7%	0.90	0.9%	0.96	0.046
NEOGONIOLITHON	LACCADIVICUM	G-0574-2	-2.6%	0.81	-1.8%	0.77	0.068
NEOGONIOLITHON	LACCADIVICUM	G-0574-3	11.4%	0.71	4.2%	0.85	0.052
AMPHIROA	CRASSA	G-0575-1	0.0%	0.81	-6.0%	0.50	0.082
AMPHIROA	CRASSA	G-0575-2	11.3%	0.14	0.9%	0.61	0.078
AMPHIROA	CRASSA	G-0575-3	-13.4%	0.11	-7.7%	0.17	0.103
PLOCAMIUM		G-0579-1	8.8%	0.44	-4.7%	0.74	0.089
PLOCAMIUM		G-0579-2	-10.6%	0.27	-7.3%	0.40	0.262
PLOCAMIUM		G-0579-3	10.4%	0.45	0.8%	0.98	0.170
DASYA		G-0585-1	15.6%	0.26	7.9%	0.80	0.253
DASYA		G-0585-2	-1.8%	0.26	1.8%	0.87	0.746
DASYA		G-0585-3	-1.7%	0.60	3.5%	0.31	0.322

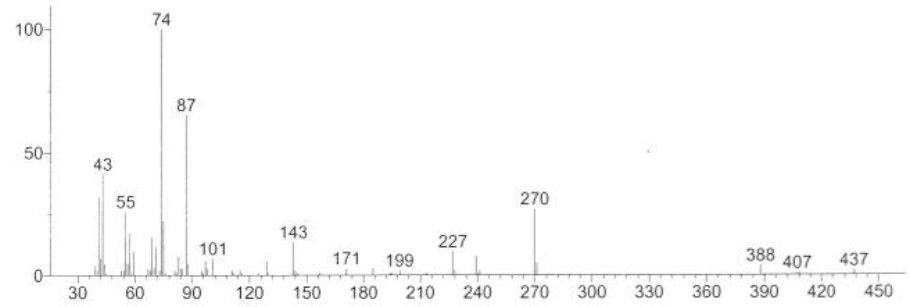
Extract Genus	Extract Species	Sample ID#	Percent Life Extension of 2 µg/mL Dose	P Value of 2 µg/mL Dose	Percent Life Extension of 10 µg/mL Dose	P Value of 10 µg/mL Dose	AO Efficacy
AMPHIROA	TRIBULUS	G-0588-1	-6.4%	0.99	12.7%	0.23	0.145
AMPHIROA	TRIBULUS	G-0588-2	1.0%	0.74	8.9%	0.60	0.256
AMPHIROA	TRIBULUS	G-0588-3	8.8%	0.22	11.0%	0.60	0.184
GALAXAURA	RUGOSA	G-0589-1	11.9%	0.59	0.0%	0.70	0.115
GALAXAURA	RUGOSA	G-0589-2	-12.7%	0.09	-12.9%	0.85	0.130
GALAXAURA	RUGOSA	G-0589-3	-5.5%	0.53	-8.0%	0.36	0.117
ACTINOTRICHIA		G-0590-1	-18.6%	0.34	7.3%	0.49	0.119
ACTINOTRICHIA		G-0590-2	-3.5%	0.87	-2.7%	0.78	0.164
ACTINOTRICHIA		G-0590-3	-6.0%	0.89	-3.7%	0.63	0.008
AMANSIA	RHODANTHA	G-0591-1	-7.6%	0.60	0.9%	0.44	0.025
AMANSIA	RHODANTHA	G-0591-2	-16.7%	0.02	-0.9%	0.62	0.021
AMANSIA	RHODANTHA	G-0591-3	4.9%	0.49	-3.0%	0.83	0.221
HYDROLITHON		G-0621-1	-14.6%	0.34	-18.2%	0.52	0.012
HYDROLITHON		G-0621-2	4.3%	0.80	0.0%	0.64	0.010
HYDROLITHON		G-0621-3	19.1%	0.18	-2.0%	0.49	0.012
PREDAEA		G-0676A-1	15.3%	0.31	-5.3%	0.54	0.144
PREDAEA		G-0676A-2	0.9%	0.85	18.8%	0.14	0.176
PREDAEA		G-0676A-3	3.2%	0.55	0.0%	0.71	0.167
ASPARAGOPSIS-PR		G-0694-1	25.5%	0.10	11.2%	0.46	0.160
ASPARAGOPSIS-PR		G-0694-2	3.9%	0.67	-13.5%	0.41	0.190
ASPARAGOPSIS-PR		G-0694-3	-4.9%	0.99	8.8%	0.88	0.216
GRACILARIA		G-0696-1	-10.4%	0.21	10.7%	0.26	0.171
GRACILARIA		G-0696-3	-7.7%	1.00	0.0%	0.69	0.145
PORTIERIA	HORNEMANNII	G-0700-1	-9.5%	0.66	1.9%	0.88	0.264
PORTIERIA	HORNEMANNII	G-0700-2	-1.0%	0.65	-3.6%	0.78	0.210
GRACILARIA		G-0793A-1	6.3%	0.89	-5.1%	0.94	0.143
GRACILARIA		G-0793A-2	0.0%	0.94	-1.0%	0.49	0.036
GRACILARIA		G-0793A-3	7.2%	0.84	-6.2%	0.96	0.037
NEURYMENIA	FRAXINIFOLIA	G-0803-1	9.1%	0.60	-3.1%	0.96	0.832
NEURYMENIA	FRAXINIFOLIA	G-0803-2	-6.4%	0.82	7.5%	0.63	0.117
NEURYMENIA	FRAXINIFOLIA	G-0803-3	-16.0%	0.11	22.5%	0.07	0.062
TRICLEOCARPA	CYLINDRICA	G-0806-1	1.0%	0.60	1.1%	0.82	0.946
TRICLEOCARPA	CYLINDRICA	G-0806-2	-10.4%	0.13	-8.8%	0.11	0.266
GELIDIUM	REEDIAE	G-0807-1	-17.7%	0.11	-4.0%	0.42	1.468
GELIDIUM	REEDIAE	G-0807-3	4.6%	0.69	8.5%	0.15	0.038
PHACELOCARPUS		G-0808-1	18.2%	0.12	3.4%	0.50	0.198
PHACELOCARPUS		G-0808-2	10.3%	0.37	-9.5%	0.15	0.104
PHACELOCARPUS		G-0808-3	-9.7%	0.19	-5.8%	0.87	0.231
UNKNOWN RHODOPHYCOTA		G-0809-1	-21.1%	0.05	-5.6%	0.53	0.025
UNKNOWN RHODOPHYCOTA		G-0809-2	-13.9%	0.33	-7.1%	0.28	0.048
UNKNOWN RHODOPHYCOTA		G-0809-3	6.5%	0.21	11.2%	0.34	0.058
AMPHIROA		G-0810-1	-25.8%	0.04	0.0%	0.99	0.217
AMPHIROA		G-0810-3	3.6%	0.56	-3.7%	0.84	0.151
JANIA-PR		G-0812-1	-22.2%	0.01	18.2%	0.09	0.331
JANIA-PR		G-0812-2	0.0%	0.86	1.0%	0.81	0.101
JANIA-PR		G-0812-3	0.0%	0.47	22.0%	0.19	0.106
GELIDIOPSIS		G-0813-1	-1.7%	0.69	-5.6%	0.64	0.021
GELIDIOPSIS		G-0813-2	-23.2%	0.01	-3.8%	0.54	0.109
GELIDIOPSIS		G-0813-3	-2.6%	0.40	-11.5%	0.22	0.138
CORYNOCYSTIS	PROSTRATA	G-0814-1	14.9%	0.21	-14.4%	0.64	0.061
CORYNOCYSTIS	PROSTRATA	G-0814-2	-18.1%	0.18	14.0%	0.09	0.241
CORYNOCYSTIS	PROSTRATA	G-0814-3	-15.8%	0.13	-4.2%	0.93	0.236
UNKNOWN RHODOPHYCOTA		G-0816A-1	4.7%	0.34	11.6%	0.22	0.055
UNKNOWN RHODOPHYCOTA		G-0816A-2	-6.8%	0.08	16.7%	0.22	0.093
UNKNOWN RHODOPHYCOTA		G-0816A-3	1.0%	0.55	3.3%	0.97	0.512
HALICHRYSIS		G-0817-1	11.8%	0.39	-12.6%	0.28	0.136
HALICHRYSIS		G-0817-2	0.0%	0.98	-5.8%	0.67	0.231
HALICHRYSIS		G-0817-3	-16.2%	0.06	-6.1%	0.64	0.129
HALYMENIA		G-0819-1	-12.9%	0.25	-10.8%	0.41	0.490
HALYMENIA		G-0819-2	-0.9%	0.47	5.2%	0.49	0.537
HALYMENIA		G-0819-3	-8.2%	0.18	-15.2%	0.05	0.531
CHEILOSPORUM		G-0820-1	21.4%	0.06	0.0%	0.98	0.136
CHEILOSPORUM		G-0820-2	-0.8%	0.84	-0.8%	0.77	0.207
CHEILOSPORUM		G-0820-3	5.3%	0.48	-11.6%	0.31	1.082
DASYPHILA		G-0821-1	-0.9%	0.45	0.9%	0.84	0.141
DASYPHILA		G-0821-2	6.8%	0.91	7.8%	0.45	0.692
DASYPHILA		G-0821-3	4.5%	0.62	-3.4%	0.84	0.240
LIAGORA		G-0822-1	9.3%	0.54	-12.7%	0.50	0.126
LIAGORA		G-0822-2	-11.5%	0.14	-5.6%	0.64	0.156

Antioxidant (AO) Efficacy is measured as the trolox equivalent antioxidant capacity as reported on p. 8.

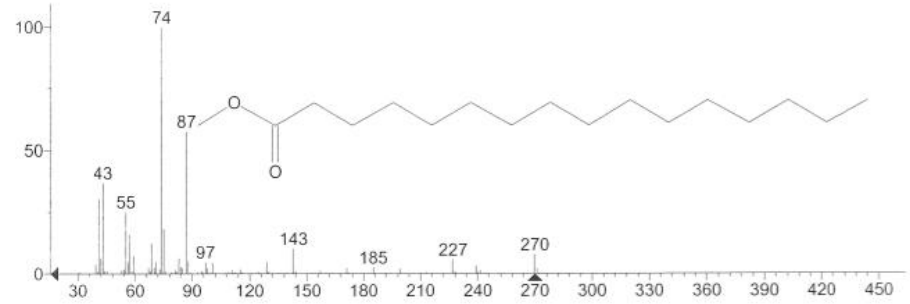
APPENDIX B

MASS SPECTROMETRY OF FATTY ACIDS

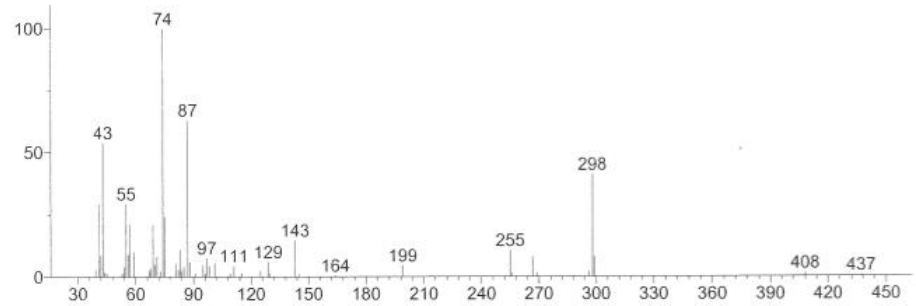
Unknown 1



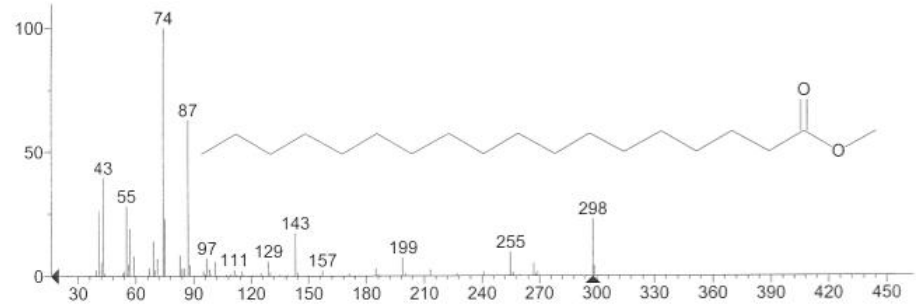
Hit 1 : Hexadecanoic acid, methyl ester
C17H34O2; MF: 872; RMF: 909; Prob 75.1%; CAS: 112-39-0; Lib: replib; ID: 9050.



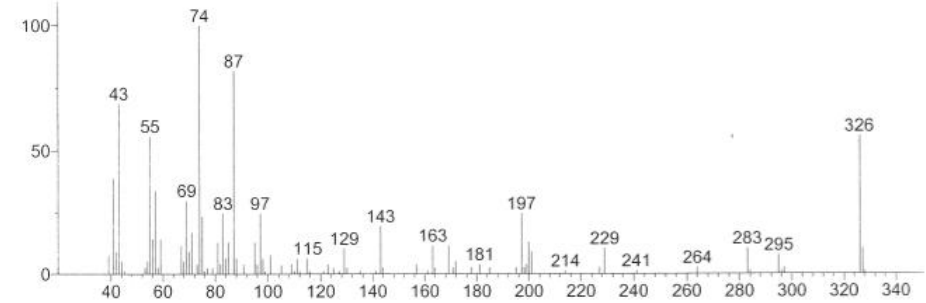
Unknown: 2



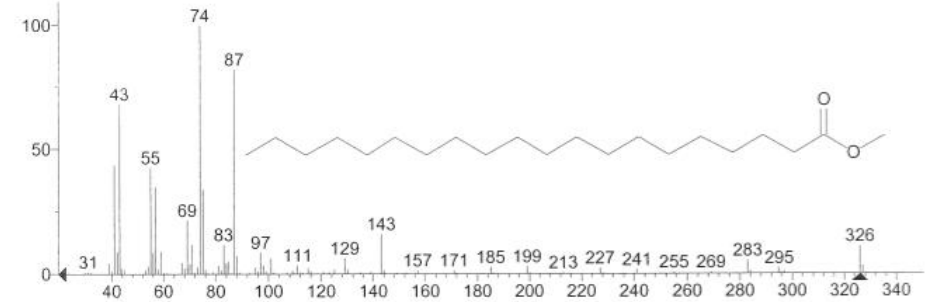
Hit 1 : Octadecanoic acid, methyl ester
C19H38O2; MF: 858; RMF: 887; Prob 80.8%; CAS: 112-61-8; Lib: replib; ID: 9088.



Unknown: 3



Hit 1 : Eicosanoic acid, methyl ester
C21H42O2; MF: 751; RMF: 834; Prob 37.1%; CAS: 1120-28-1; Lib: replib; ID: 9055.



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